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(71) Applicant: **DANA-FARBER CANCER INSTITUTE, INC.** [US/US]; 44 Binney Street, Boston, MA 02115 (US).

(72) Inventors: **DEPINHO, Ronald, A.**; 565 Boylston Street, Brookline, MA 02445 (US). **CHIN, Lynda**; 565 Boylston Street, Brookline, MA 02445 (US).

(74) Agent: **CLARK, Paul, T.**; Clark & Elbing LLP, 176 Federal Street, Boston, MA 02110-2214 (US).

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(54) Title: INDUCIBLE CANCER MODEL TO STUDY THE MOLECULAR BASIS OF HOST TUMOR CELL INTERACTIONS IN VIVO

(57) Abstract: A non-human mammal having incorporated into its genome an expression construct in which nucleic acid encoding and oncogene is operably linked to an inducible promoter; the mammal further has a genetic mutation that causes it to have an increased susceptibility to cancer.

INDUCIBLE CANCER MODEL TO STUDY THE MOLECULAR
5 # BASIS OF HOST TUMOR CELL INTERACTIONS IN VIVO

Background of the Invention

Advanced malignancy represents the phenotypic end-point of successive genetic lesions that impact on the function and regulation of oncogenes and tumor suppressor genes[1]. The established tumor is maintained through complex and poorly

10 understood host-tumor interactions guiding processes such as angiogenesis and immune sequestration. The numerous and diverse genetic alterations that accompany tumor- ogenesis raises questions as to whether experimental cancer-promoting mutations remain relevant to tumor maintenance.

Summary of Invention

15 Melanoma genesis and maintenance were shown to be strictly dependent upon H-RAS^{V12G} expression in a novel doxycycline-inducible H-RAS^{V12G} INK4a null mouse melanoma model. Withdrawal of doxycycline and H-RAS^{V12G} down-regulation resulted in clinical and histological regression of primary and explanted tumors. The initial stages of regression were highlighted by dramatic

20 activation of apoptosis in the tumor cell and host-derived endothelial cells. Although the regulation of VEGF was found to be RAS-dependent *in vitro*, the failure of persistent endogenous and enforced VEGF expression to sustain tumor viability indicated that the tumor maintenance actions of activated RAS extend beyond the regulation of VEGF gene expression *in vivo*. Together these data provide genetic

25 evidence that H-RAS^{V12G} plays a critical role in both the genesis and maintenance of solid tumors.

Accordingly, the invention features a non-human mammal having incorporated into its genome an expression construct including nucleic acid encoding an oncogene operably linked to an inducible promoter, the mammal

30 further having a genetic mutation that causes it to have a greater susceptibility

to cancer than a mammal not having that mutation.

In preferred embodiments: the mammal is a mouse; the oncogene is ras; the ras gene has an activating mutation; the inducible promoter can be induced by doxycycline; and the mutation is in DNA encoding a tumor
5. suppressor such as INK4.

In a second, related aspect, the invention features a non-human mammal having incorporated into its genome: (i) a first expression construct in which a reverse tetracycline transactivator is operably linked to a tissue-specific promoter; and (ii) a second expression construct in which nucleic acid encoding
10 an oncogene is operably linked to a promoter that can be regulated by the reverse tetracycline transactivator; the mammal further has a genetic mutation that causes it to have greater susceptibility to cancer than a mammal that does not have the mutation. In preferred embodiments of this aspect of the invention, the tissue-specific promoter is a tyrosine promoter, and expression of
15 the oncogene results in the mammal having greater susceptibility to cancer than a mammal having the mutation but not expressing the oncogene.

In a third, related aspect, the invention features a non-human mammal having incorporated into its genome: (i) a first expression construct in which a reverse tetracycline transactivator is operably linked to a tyrosinase
20 promoter; and (ii) a second expression construct in which nucleic acid encoding ras is operably linked to a promoter than can be regulated by the reverse tetracycline transactivator.

The invention can be used as a model for any cancer, and in particular solid tumors such as melanoma. The oncogene linked to the
25 inducible promoter and/or the mutation in the genome of the mammal are chosen to direct development of the desired tumor type.

Brief Description of the Drawings

Figure 1. Inducible Tyr/Tet-RAS transgenic mice on INK4a deficient background developed cutaneous melanomas.

    A.   Summary of tumor incidence in the Tyr/Tet-RAS - INK4a null colony and impact of doxycycline treatment.

    B.   Left: anti-TRP1 staining of a primary cutaneous melanoma. Note strong immunoreactivity in dermal nodule with overlying intact epidermis; e=epidermis; d=dermis. Right: in situ hybridization for H-RAS^{V12G} transcript in a primary melanoma nodule.

Figure 2. Activated RAS expression is necessary to maintain growth of established cutaneous melanomas in vivo.

    A.   Tumor from R348 (Tet-RAS founder line #65) was measured at intervals and longest dimension was plotted against time. Time 0 represents initial induction with doxycycline. Red brackets indicate periods during which the animal was ON doxycycline induction. Blue lines indicate period without doxycycline administration. Insets: Representative photographs of R348 tumor taken on respective time points (ON and 12 days OFF) during the first cycle of doxycycline treatment. All primary melanomas (n=10) exhibited a strong regression response following doxycycline withdrawal, although 2 of the largest tumors recurred at the site as slow growing tumors of indeterminate type (see text). Two fully regressed tumor-bearing mice were re-induced to form large tumors, one of which regressed fully upon doxycycline withdrawal (depicted here), while growth of the other tumor remained stable for additional 10 days before sacrifice.

    B.   Regression of primary melanoma in R767. (a) = ON, (b) = 3 days and (c) = 10 days OFF.

    C.   Photomicrographs of H&E sections of one of the tumors in R348 measured and depicted in A. Top panel 200X, lower panel 400X magnification. ON = histology of the tumor biopsied while on doxycycline. Note anaplastic and pleiomorphic large cells. 48 Hr OFF = Histology of the tumor biopsied 48 hours after

withdrawal of doxycycline. Note more orderly spindle morphology. 12 D OFF = histology of the site where tumor was biopsied 12 days after withdrawal of doxycycline. Note intact epidermis and scattered foci of transformed cells (arrows). * denotes hair follicle; e=epidermis.

5   D.   Photomicrographs of TUNEL (top panel) staining of tumors from mouse R405 (Tet-RAS founder line #72) biopsied ON (left) and 72 Hrs OFF doxycycline (middle). Higher power magnification (right) of the bracketed region in middle. Arrow = TUNEL positivity in endothelial cell. Middle panel shows Ki67 immunostaining for detection of S phase nuclei. Bottom panel depicts anti-CD34

10   staining patterns of the same tissues.

Figure 3. In vitro and in vivo behavior of melanoma cells in the presence and absence of doxycycline.

A.   Photomicrograph of SCID mice maintained on (a) doxycycline and off (b) doxycycline after subcutaneous injection of R192 cells. Arrows: subcutaneous

15   tumors. Similar experiments performed with multiple independently derived melanoma cell lines were summarized in the table. "Duration" refers to the period of observation in months.

B.   Growth curve determinations of three independently derived melanoma cell lines with and without doxycycline supplementation in media (see

20   Methods). Solid lines: doxycycline-treated. Dotted lines: doxycycline-free. Note cultures were subconfluent during the first 5 days.

Figure 4. VEGF is not sufficient to sustain tumor viability following doxycycline withdrawal.

A.   RAS activity levels after withdrawal from doxycycline in vivo and

25   in vitro. R545 melanoma cells in cultures or R545 derived SCID tumors were subjected to Raf-GST pull down assay (see Methods) to determine level of RAS activity at indicated time after doxycycline withdrawal. For in vivo assay, two independently derived samples were assayed except for the 0 hour time point.

B.   Endogenous VEGF protein levels of cultured R545 and R673 cells (Left), as well as SCID derived R545 tumors (Right).

C.   Enforced VEGF expression in R545 melanoma cells is not sufficient to block tumor regression in SCID mice after doxycycline withdrawal. Transduced cells were injected on day -11 and doxycycline was withdrawn from drinking water on day 0.

D.   Photomicrographs of H&E and TUNEL stainings of SCID tumors from C. Note large ectactic vascular spaces in VEGF-transduced tumors. Increase in # of TUNEL positive nuclei (both tumor and endothelial cells) was evident in both 48 hours and 72 hours after doxycycline withdrawal in tumors with or without enforced VEGF expression. Arrows: TUNEL positive endothelial cells lining vascular spaces.

Detailed Description of the Invention

To develop a cancer model in which dominantly acting oncoproteins are somatically regulated in vivo, transgenic mouse lines harboring the reverse tetracycline transactivator[2] under the control of the tyrosinase gene promoter-enhancer elements (designated Tyr-rtTA) and another containing the H-RAS^{V12G} open reading frame driven by a minimal promoter containing multimerized tet-operons [2,3] (designated Tet-RAS) were inter-crossed with INK4a +/- mice to generate cohorts of single and double transgenic mice (designated Tyr/Tet-RAS) homozygous null for INK4a. Upon weaning, a subset of single and double transgenic INK4a -/- mice were administered doxycycline in the drinking water [4]. In the doxycycline-treated group, 10 of 40 Tyr/Tet-RAS INK4a-/- mice developed melanomas with an average latency of 60 days (Fig. 1A). In contrast, the untreated Tyr/Tet-RAS INK4a-/- mice (n=12) or treated Tet-RAS INK4a-/- mice (n=23) failed to develop melanomas. The Tyr/Tet-RAS INK4a-/- melanomas shared all of the macroscopic features of the constitutive Tyr-RAS INK4a-/- melanomas 5, manifesting as amelanotic, invasive and highly vascular tumors - features reminiscent of nodular-type melanoma in humans (for example, see Fig. 2A&B). Histological examination revealed a spindle morphology with anaplastic and pleiomorphic cytology (Fig. 2C), strong

immunoreactivity to the early melanocyte-specific marker tyrosinase-related protein-1 (TRP-1) [6], and robust H-RAS^{V12G} expression and activity in tumors and tumor-derived cell lines in culture (Fig 1B and Fig. 4A).

5

While these and previous results [5,7] confirm a causal role for H-RAS^{V12G} in melanoma development, the capacity to regulate H-RAS^{V12G} activity in these melanomas provided an unprecedented opportunity to determine what role, if any, activated RAS plays in tumor maintenance. To this end, different doxycycline-treated Tyr/Tet-RAS INK4a-/- mice bearing one or multiple independent primary melanomas, ranging in size from 0.5 to 1.5 cm in diameter, were withdrawn from doxycycline

10

administration (Fig 2A and 2B). Following an initial loss of tumor erythema (Fig. 2B, compare b to a), these large tumors regressed to barely detectable or undetectable masses with only residual scattered tumor foci on microscopic examination by day 14 of doxycycline withdrawal (Fig. 2C, compare right panel with left). Consistent with the presence of residual microscopic disease, re-administration of doxycycline

15

resulted in the rapid recurrence of tumor at the site of the previous tumor (Fig. 2A). As such, this model may find use in the development of anti-oncologics directed towards minimal residual disease. All primary melanomas (n=10) exhibited regression upon doxycycline withdrawal; however, doxycycline-independent tumors can persist or re-emerge (n=3) particularly with large tumor burdens (See Fig. 2

20

legend). These latter tumors were whitish, exhibited a less anaplastic spindle cytology, failed to express the H-RAS^{V12G} transgene by Western blot analyses, and were TRP-1 negative (data not shown). These data suggest that these doxycycline-independent tumors are either a different cancer type such as fibrosarcoma or RAS-independent melanomas.

25

Although these results and those of others [8] underscore a role for RAS in tumorigenesis, this novel model system permits an investigation of mechanisms underlying tumor regression in vivo. To this end, we first assessed the impact of doxycycline withdrawal in different Tyr/Tet-RAS INK4a-/- tumor derived cell lines in cell culture. Melanoma cell lines were established from primary tumors in

30

doxycycline-supplemented media and were shown to express H-RAS^{V12G} mRNA,

protein, and activity in a doxycycline-dependent manner (Fig. 4A and data not

shown). The derivative melanoma cell lines remained doxycycline-responsive as

shown by their strict doxycycline-dependency for tumorigenic potential in SCID mice.

Specifically, subcutaneous injection of purified Tyr/Tet-RAS INK4a-/- melanoma cell

5   lines yielded tumors in SCID mice treated with doxycycline with 100% efficiency,

while those mice not receiving doxycycline remained tumor-free after many months

of observation (Fig. 3A). In cell culture, the presence or absence of doxycycline did

not significantly influence the subconfluent growth rates of multiple independently

derived lines (Fig. 3B). Similarly, the presence of doxycycline failed to confer

10   enhanced growth potential in low serum conditions (0.5% and 1% FCS) (data not

shown). However, the presence of doxycycline modestly enhanced the growth in

dense cultures, suggesting that in vivo RAS may provide a continued cell-autonomous

advantage for tumor growth. On the other hand, the data is also consistent with the

possibility that tumor regression following doxycycline withdrawal may reflect a

15   continued requirement for RAS in promoting and sustaining non-cell-autonomous

tumor-host interactions that are essential for tumor growth and maintenance.

One hypothesis supporting the latter possibility is that continued

expression of RAS confers on the tumor cells the capacity to evade host immune

response [9]; thus tumor regression occurs due to onset of immune rejection by the host.

20   To examine the contribution of an intact immune system to tumor regression, we

determined the regression kinetics of tumors derived from purified Tyr/Tet-RAS

INK4a-/- melanoma cell lines in SCID tumor explants. Upon doxycycline

withdrawal, established tumors exhibited rapid reductions in tumor size accompanied

by histological, proliferative and apoptotic profiles that were indistinguishable from

25   that of the primary tumors in immune competent hosts (data not shown). As SCID

mice lack functioning B and T lymphocytes (though they maintain functional natural

killer cells and macrophages), these data are consistent with the view that the immune

system does not play a principal role during the initial phase in which the bulk of the

tumor burden is eliminated.

Another important aspect of tumor reliance on host interactions involves angiogenic support. In fact, examination of regressing tumors suggested that vascular regression might even precede frank tumor loss. Serial biopsies of the same primary Tyr/Tet-RAS INK4a-/- tumors were performed following doxycycline withdrawal at

5   times 0 hours, 24 hours, 48 hours and 72 hours. Tumor-bearing mice from both Tet-RAS founder lines #65 and #72 were examined (e.g. R348 and R405 from line #65 and #72, respectively) and yielded identical phenotypes. Upon microscopic examination, a morphologic transition from severe anaplasia to a more organized spindle cytoarchitecture occurred by 48 hours post doxycycline withdrawal (R348

10   tumor shown in Fig. 2C). A modest decline in proliferative index and a dramatic increase in apoptosis (R405 tumor shown in Fig 2D) accompanied these histological changes. In many primary tumors examined, the apoptotic response was evident as early as 24 hours post doxycycline withdrawal (data not shown). Most notably, many TUNEL positive cells were found to reside in close proximity to small tumor vessels,

15   specifically the cells comprising the vessel wall (Fig. 2D). Vessel-associated apoptosis coincided with a marked decrease in CD34 and CD31 immunoreactivity, two classical markers of the endothelial cell [10,11] (Fig. 2D, CD31 not shown).

The significant apoptotic rate of cells lining tumor vessels suggested that sustained activated RAS expression may be required for the critical host-tumor

20   symbiotic interaction that sustains stable tumor vasculature. Consistent with this, previous cell culture data have shown that oncogenic K- and H-RAS^{V12G} can stimulate VEGF gene expression [12-14]. To determine the role, if any, of VEGF in RAS-dependent tumor maintenance, RAS activity as measured by the RAF-GST pull-down assay and VEGF protein levels (see Methods) were serially determined in

25   cell culture and tumor explants following doxycycline withdrawal. In cell culture, a decline in H-RAS^{V12G} activity levels correlated with down-regulation of VEGF expression (Fig. 4A&B). In the tumor explants, although a decline in RAS activity led to an eventual decrease in VEGF levels during advanced stages of tumor regression (post day 3; Fig. 4A and 4B), early regression and endothelial cell

30   apoptosis occurred in the face of sustained VEGF levels, presumably due to

hypoxic/anoxic stimuli [15-18]. The contrasting VEGF kinetics suggested that tumor regression in vivo is not mediated by the loss of RAS-stimulated VEGF expression and that high VEGF levels are insufficient to maintain tumor viability in the absence of H-RAS^{V12G} expression. To examine this issue more directly, the

5   doxycycline-responsive R545 melanoma cell line was engineered to constitutively express either GFP (green fluorescence protein) or VEGF plus GFP. The VEGF-transduced cell lines expressed on average 10-fold higher levels of VEGF than the GFP-transduced control in the absence of doxycycline (10 ng/ml versus < 1 ng/ml by ELISA). Although the VEGF-transduced cells exhibited a more rapid tumor

10   growth, doxycycline withdrawal resulted in regression of these tumors despite high level of enforced VEGF expression (Fig. 4C). Furthermore, as in primary tumors and SCID tumors derived from the parental untransduced melanoma lines, this tumor regression was associated with marked activation of apoptosis in both tumor cells and host endothelial cells (Fig. 4D).

15   Taken together, these data demonstrate that, while activated RAS regulates VEGF gene expression in tumor cells as previously reported [13,19-21], complex and poorly understood RAS-independent mechanisms contribute significantly to the regulation of VEGF in established tumors upon doxycycline withdrawal. Correspondingly, the fact that initiation of tumor regression and associated vascular

20   collapse take place in the face of either robust endogenous or retrovirally-enforced VEGF expression indicates that VEGF is not sufficient for tumor maintenance, consistent with previous findings [14], and that the role of activated RAS in tumor maintenance extends beyond the regulation of VEGF gene expression. Thus, the in vivo model reported here will serve to uncover additional aspects of the complex

25   homotypic and heterotypic interactions between host and tumor cells essential for the maintenance of fully formed solid tumors. While an improved understanding of such interactions could stimulate the development of therapeutics directed toward the less plastic host cell compartment, it is notable that despite the occurrence of many genetic changes in these tumors, effective anti-RAS therapeutics would be predicted to hold

30   significant promise in an anti-cancer armamentarium.

Methods

Production of the transgenic mice. The reverse tetracycline

transactivator (rtTA) is a 1050 bp EcoRI/BamHI fragment isolated from

pUHD172-1neo [2]. The Tet promoter contains a XhoI/EcoRI fragment of CMV

5   minimal promoter linked to the tet operator sequences [2]. The tyrosinase

enhancer/promoter and the H-RAS^{Val12} transgene were as described elsewhere [3,5,22].

Multiple founder lines were generated for both transgenes at the expected frequencies.

One activator line (Tyr/rtTA, #37) and two independent reporter lines (Tet-RAS, #65

and #72) were utilized for these studies.

10   **Primary tumors, derivative cell lines and SCID explant tumors.**

Transgenic mice were fed doxycycline drinking water (2 mg/ml sucrose water) and

observed for spontaneous tumor development. Primary tumors were adapted to

culture by mechanical mincing with sterilized razor blades and brief trypsinization

and maintained on RPMI media containing 10% serum and supplemented with

15   doxycycline (2 μg/ml media) [4]. For the SCID explant tumors, 2 -5 1x10^6 established

melanoma cells were injected SQ into the flanks of adult SCID mice maintained on

doxycycline or regular drinking water. These cell lines were passaged sufficiently to

insure elimination of immunocytes from the original host.

RNA and protein analysis. Total RNAs were isolated from tumor tissues

20   and cells using Trizol reagents (Gibco BRL). For H-RAS^{V12G}, a 700 bp SalI cDNA

fragment isolated from pBABE-RAS (kind gift of Scott Lowe) was used as probe.

For VEGF, a SacI/Kpn1 600 bp ORF fragment was isolated from mVEGF.KS (kind

gift of P. Maisonpierre). RNA in situ hybridization was performed as described

elsewhere [23] on tumor samples snap-frozen in OCT (Lab-Tek). Western blots of

25   tumor or cell lysates (100 μl input) were run on 15% SDS-PAGE gel and probed with

Anti RAS MAB (Oncogene). RAS activity was determined by RAS Activation Assay

(Upstate Biotechnology) as per manufacture's protocol. VEGF protein levels were

determined in duplicate by ELISA (R&D System) using 100 mg of protein input

(from tissue lysates or conditioned media). Microplates were ready by OPTIMax

30   tunable microplate reader (Molecular Devices) and analyzed with SoftMax Pro.

Histological analyses and immunohistochemistry. Tissue samples were formalin fixed and paraffin embedded. Anti-TRP1 staining using TA99 monoclonal antibody [6], TUNEL, Ki67 and Anti-CD34 staining were performed as described elsewhere [5,11,24,25].

5   **Growth curves.** Cells from each cell line were seeded at a density of 20,000 cells per well in a 12-well plate in media with or without doxycycline. Media was changed every 3 days for all samples. Duplicate wells were trypsinized, cell numbers counted by hemacytometer at indicated time points and plotted against time. Studies were conducted in media containing 10%, 1% and 0.5% serum. Growth curve

10   determinations were performed in cells maintained on doxycycline prior to experiments as well as cells already removed from doxycycline for 3 days.

Enforced VEGF expressing melanoma cell line. R545 melanoma cell line was transduced with LZRSpBMN-IRES-GFP and LZRSpBMN-VEGF-IRES-GFP purified populations established by FACS sorting [26].

15   SCID explant experiments with transduced cell lines were generated under the same conditions as described above. Tumor sizes were measured with tumor caliber in two dimensions and area plotted against time (in days).

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What is claimed:

1. A non-human mammal having incorporated into its genome an expression construct comprising nucleic acid encoding an oncogene operably linked to an inducible promoter, said mouse further comprising a genetic mutation that causes said mammal to have greater susceptibility to cancer than

5   a mammal not comprising said mutation.

2. The mammal of claim 1, wherein said mammal is a mouse.

3. The mammal of claim 1, wherein said oncogene is ras.

4. The mammal of claim 3, wherein said ras has an activating mutation.

10   5. The mammal of claim 1, wherein said inducible promoter can be induced by doxycycline.

6. The mammal of claim 1, wherein said mutation is in DNA encoding a tumor suppressor.

7. The mammal of claim 6, wherein said tumor suppressor is INK4.

15

8. A non-human mammal having incorporated into its genome: (i) a first expression construct comprising a reverse tetracycline transactivator operably linked to a tissue-specific promoter; and (ii) a second expression construct comprising nucleic acid encoding an oncogene operably linked to a

5 promoter that can be regulated by said reverse tetracycline transactivator, said mammal further comprising a genetic mutation that causes said mammal to have greater susceptibility to cancer than a mammal not comprising said mutation.

9. The mammal of claim 8, wherein said tissue-specific promoter is

10 a tyrosinase promoter.

10. The mammal of claim 8, wherein expression of said oncogene results in said mammal having greater susceptibility to cancer than a mammal comprising said mutation but not expressing said oncogene.

11. A non-human mammal having incorporated into its genome: (i) a

15 first expression construct comprising a reverse tetracycline transactivator operably linked to a tyrosinase promoter; and (ii) a second expression construct comprising nucleic acid encoding ras operably linked to a promoter that can be regulated by said reverse tetracycline transactivator.

A

# Mice	Genotype			Dox Induction	# Mice with Melanoma	Latency (days ± SD)
	Tyr-rtTA	Tet-Ras	INK4a			
40	+	+	−/−	Yes	10	60 ± 26
23	−	+	−/−	Yes	0	—
12	+	+	−/−	No	0	—

B
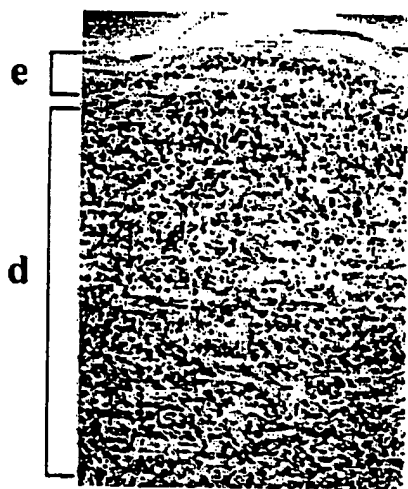
α-TRP1 IHC
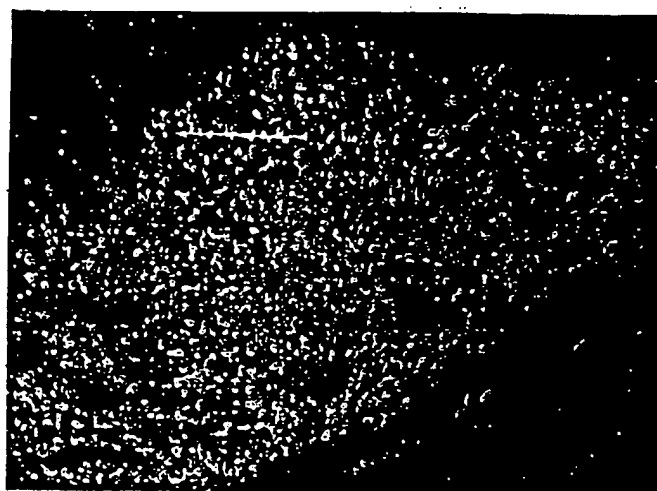
RAS in situ hybridization

FIG. 1

A

B
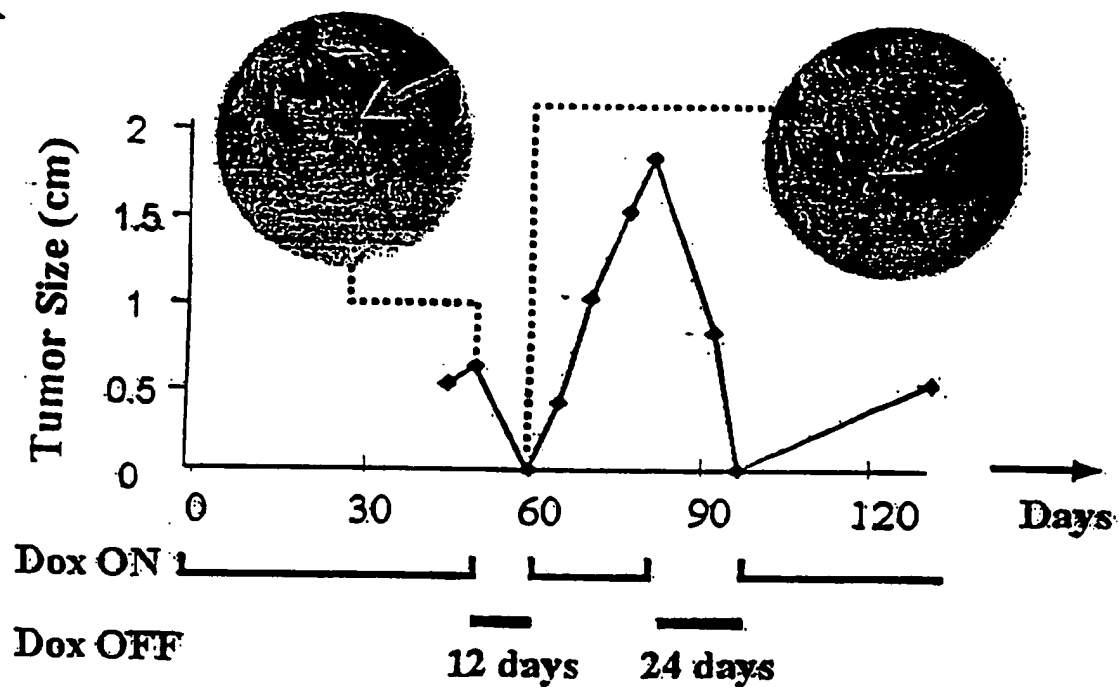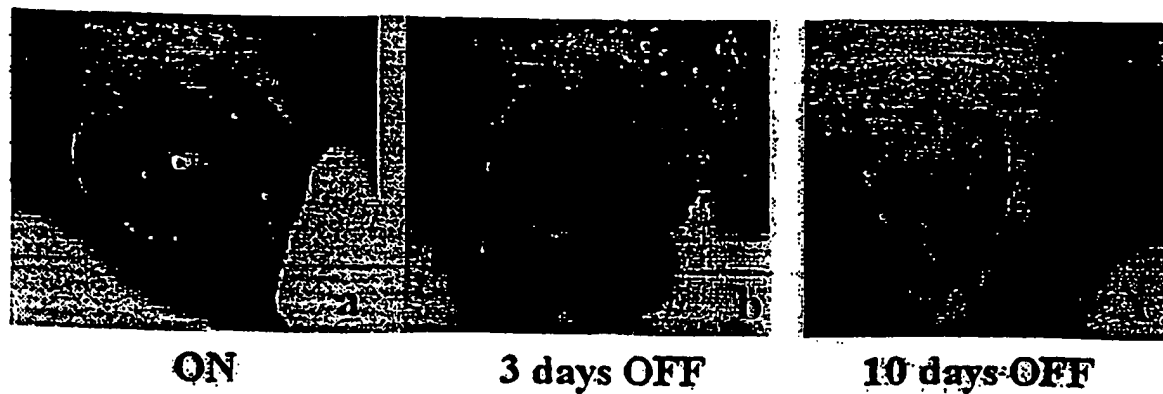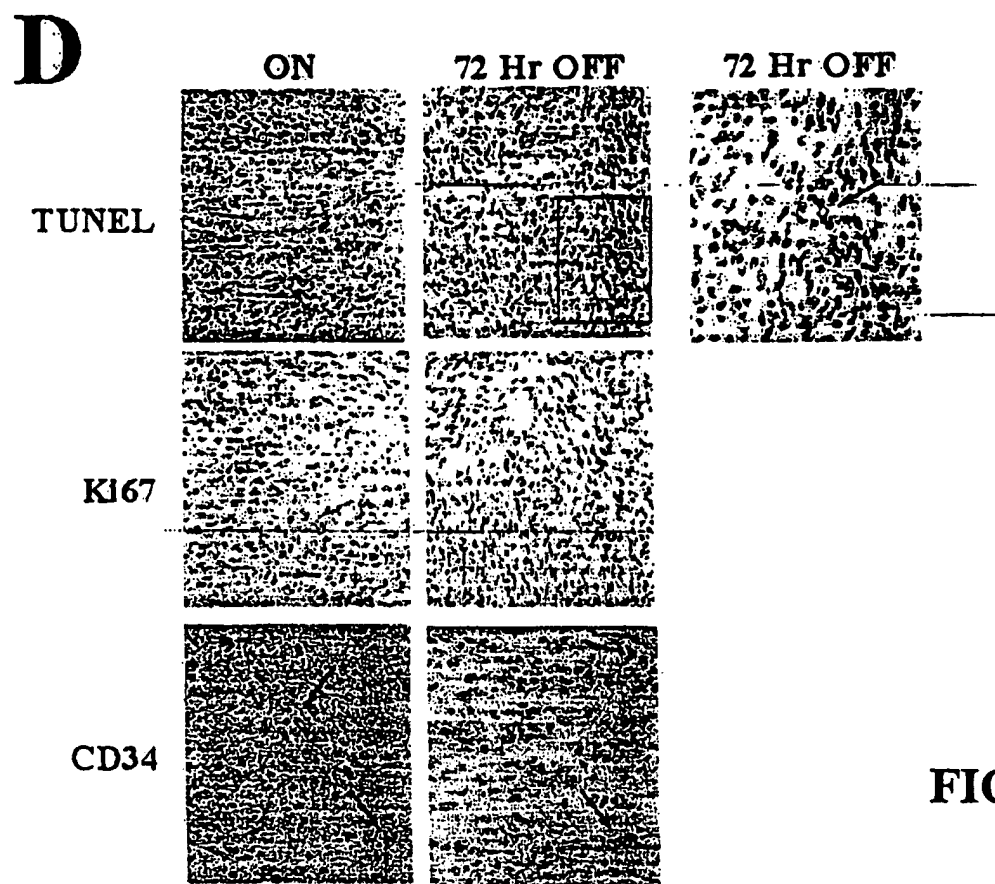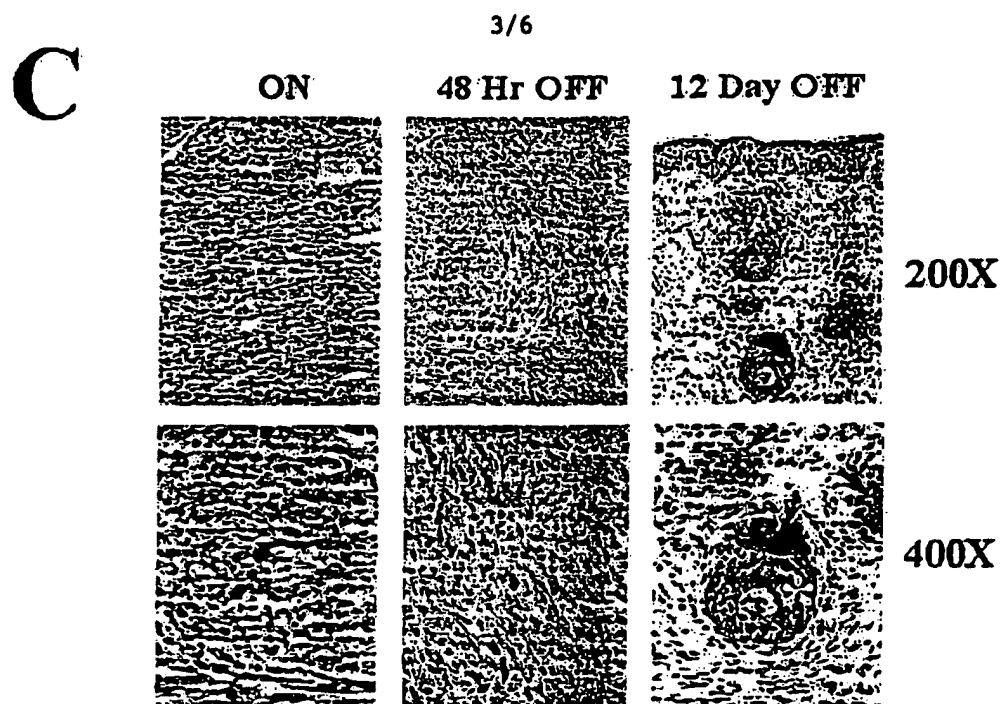
FIG. 2

FIG. 2

A
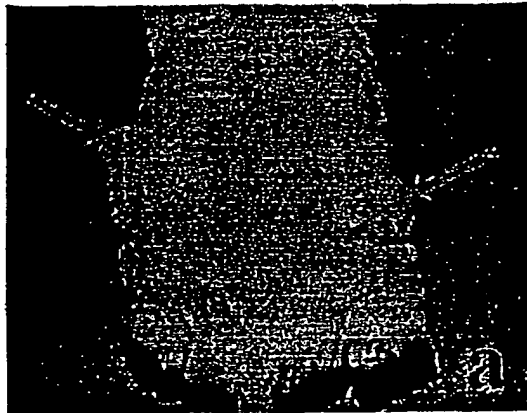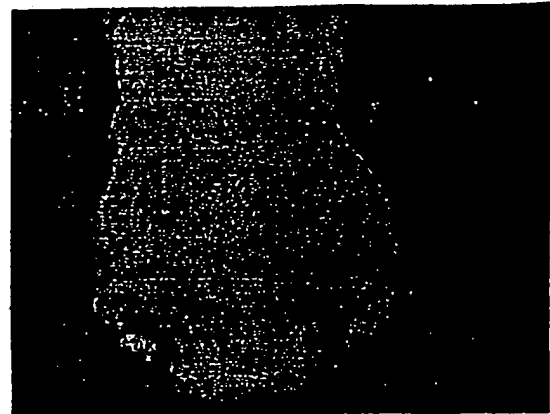
+ Doxycycline   - Doxycycline

Cell Line	+ Doxycycline		- Doxycycline		
	# SCID	Tumor	# SCID	Tumor	Duration
R192	4	4	2	0	4.5 mo
R405	3	3	1	0	1.5 mo
R545	28	28	2	0	4.0 mo
R767	20	20	1	0	3.0 mo

B
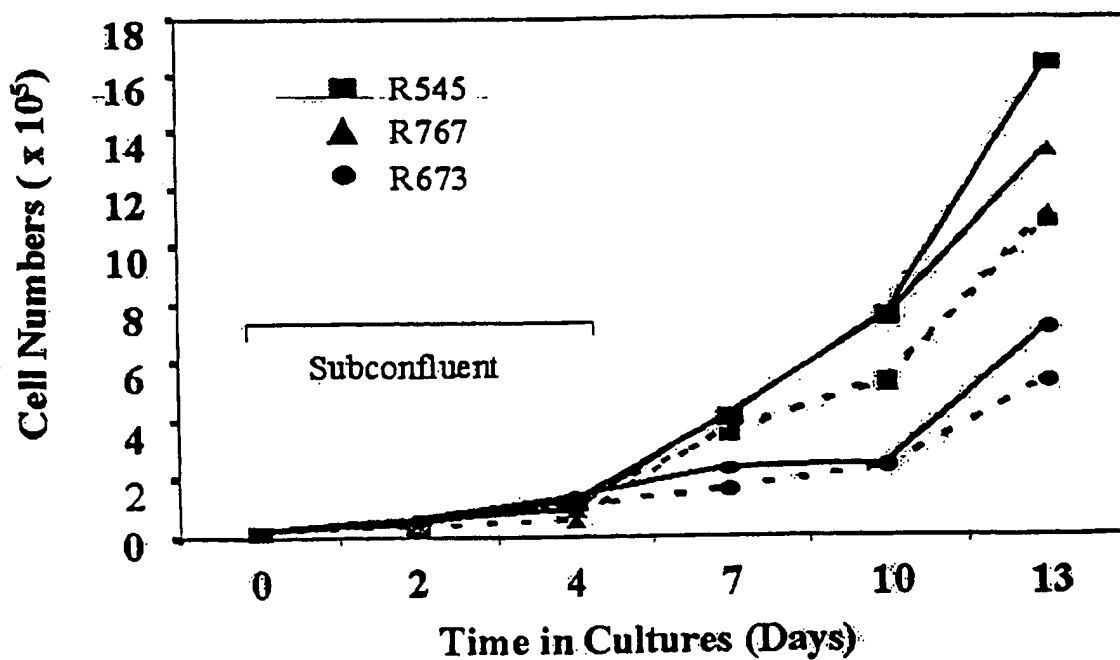
FIG. 3
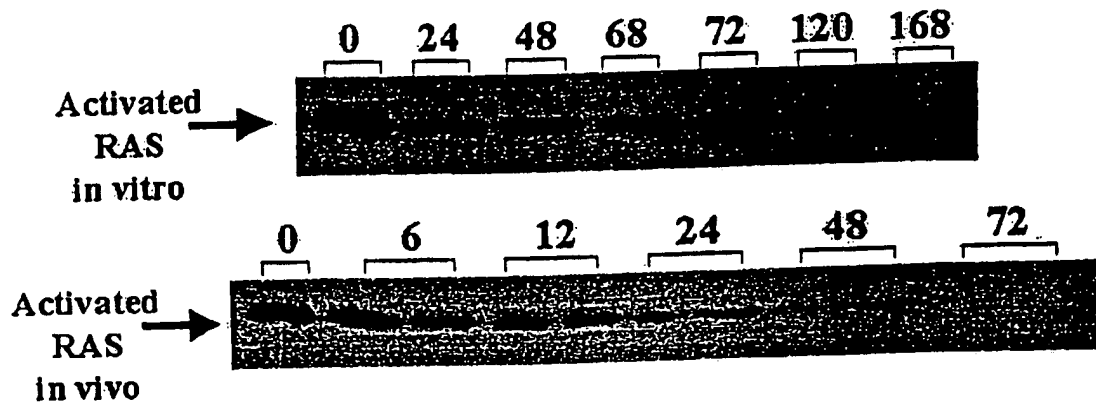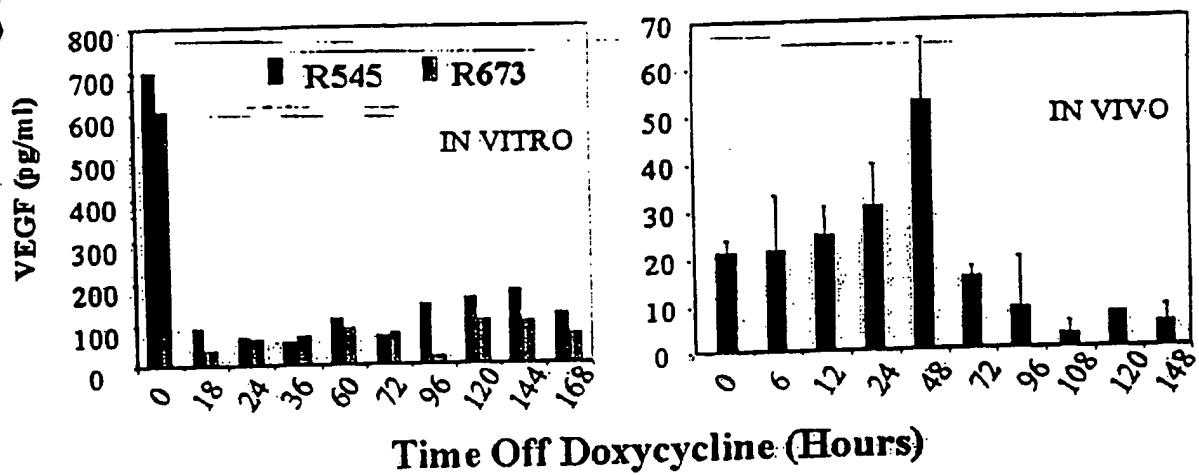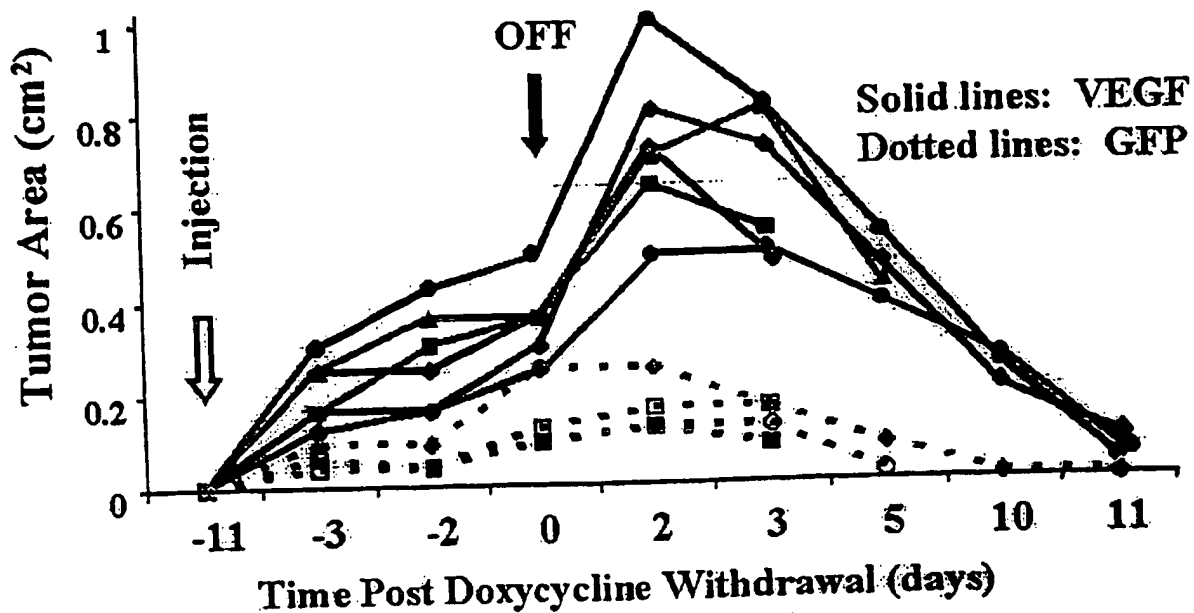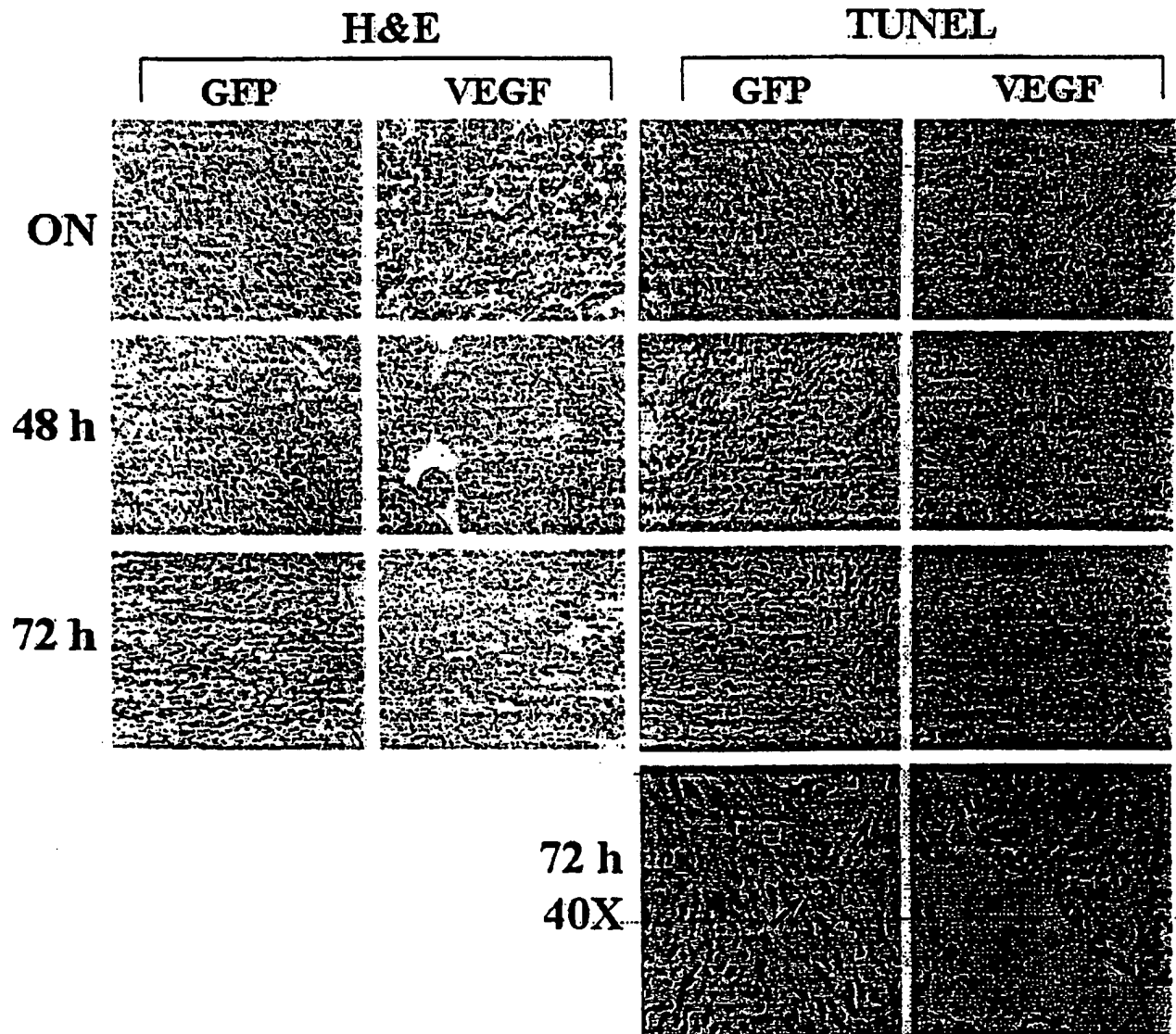
FIG. 4

D

FIG. 4

INTERNATIONAL SEARCH REPORT

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(7)   :C12N 15/09, 15/63, 15/00
US CL   :800/14, 18, 10, 21, 22, 25, 3; 435/455, 463, 467
According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   800/14, 18, 10, 21, 22, 25, 3; 435/455, 463, 467

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	CHIN et al. Flipping the oncogene switch illumination of tumor maintenance and regression. Trends in Genetics. April 2000, Vol. 16, No. 4, pages 147-150, see entire document.	1-11
Y	RUDOLPH et al. Genetically modified animals in pharmacological research: future trends. European Journal of Pharmacology. 30 June 1999, Vol. 375, Nos. 1-3, pages 327-337, see entire document.	1-11
Y	JONES et al. Analysis of Tumor Suppressor Genes Using Transgenic Mice. Methods: A Companion to Methods in Enzymology. December 1995, Vol. 8, No. 3, pages 247-258, see entire document.	1-11

[X]	Further documents are listed in the continuation of Box C.	[ ]	See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 SEPTEMBER 2000	18 OCT 2000

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer JILL D. MARTIN
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LOIDI et al. Complex Regulation of Prothymosin Alpha in Mammary Tumors Arising in Transgenic Mice. Life Sciences. 30 April 1999, Vol. 64, No. 23, pages 2125-2133, see entire document.	1-11
X - Y	BARBER et al. Potassium conductances and proliferation in conditionally immortalized renal glomerular mesangial cells frm the H-2Kb-tsA58 transgenic mouse. Biochimica et Biophysica Acta. 1997, Vol. 1355, pages 191-203, see especially page 192.	1, 2 --- 3-11
Y	VILE et al. Tissue-Specific Gene Expression from Mo-MLV Retroviral Vectors with Hybrid LTRs Containing the Murine Tyrosinase Enhancer/Promoter. Virology. 1995, Vol. 214, pages 307-313, see entire document.	1-11
Y	US 5,919,997 A (BEACH et al.) 06 July 1999, see entire document.	1-11
Y	US 5,859,310 A (BUJARD et al.) 12 January 1999, see entire document.	1-11

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

NPL, MEDLINE, BIOSIS, CAPLUS, EMBASE, WEST

search terms: inducible promoter, oncogene, INK4, ras, tumor suppressor, tetracycline, tyrosinase promoter, reverse tetracycline transactivator, transgenic